

**DIAGNOSTIC AND THERAPEUTIC USE OF A VOLTAGE-GATED ION
CHANNEL FOR NEURODEGENERATIVE DISEASES**

The present invention relates to methods of diagnosing, prognosticating and monitoring the progression of neurodegenerative diseases in a subject. Furthermore, methods of therapy control and screening for modulating agents of neurodegenerative diseases are provided. The invention also discloses pharmaceutical compositions, kits, and recombinant animal models.

Neurodegenerative diseases, in particular Alzheimer's disease (AD), have a strongly debilitating impact on a patient's life. Furthermore, these diseases constitute an enormous health, social, and economic burden. AD is the most common neurodegenerative disease, accounting for about 70% of all dementia cases, and it is probably the most devastating age-related neurodegenerative condition affecting about 10% of the population over 65 years of age and up to 45% over age 85 (for a recent review see Vickers et al., *Progress in Neurobiology* 2000, 60: 139-165). Presently, this amounts to an estimated 12 million cases in the US, Europe, and Japan. This situation will inevitably worsen with the demographic increase in the number of old people ("aging of the baby boomers") in developed countries. The neuropathological hallmarks that occur in the brains of individuals with AD are senile plaques, composed of amyloid- β protein, and profound cytoskeletal changes coinciding with the appearance of abnormal filamentous structures and the formation of neurofibrillary tangles.

The amyloid- β ($A\beta$) protein evolves from the cleavage of the amyloid precursor protein (APP) by different kinds of proteases. The cleavage by the β/γ -secretase leads to the formation of $A\beta$ peptides of different lengths, typically a short more soluble and slow aggregating peptide consisting of 40 amino acids and a longer 42 amino acid peptide, which

rapidly aggregates outside the cells, forming the characteristic amyloid plaques (Selkoe, *Physiological Rev* 2001, 81: 741-66; Greenfield et al., *Frontiers Bioscience* 2000, 5: D72-83). Two types of plaques, diffuse plaques and neuritic plaques, can be detected in the brain of AD patients, the latter ones being the classical, most prevalent type. They are primarily found in the cerebral cortex and hippocampus. The neuritic plaques have a diameter of 50µm to 200µm and are composed of insoluble fibrillar amyloids, fragments of dead neurons, of microglia and astrocytes, and other components such as neurotransmitters, apolipoprotein E, glycosaminoglycans, α 1-antichymotrypsin and others. The generation of toxic A β deposits in the brain starts very early in the course of AD, and it is discussed to be a key player for the subsequent destructive processes leading to AD pathology. The other pathological hallmarks of AD are neurofibrillary tangles (NFTs) and abnormal neurites, described as neuropil threads (Braak and Braak, *Acta Neuropathol* 1991, 82: 239-259). NFTs emerge inside neurons and consist of chemically altered tau, which forms paired helical filaments twisted around each other. Along the formation of NFTs, a loss of neurons can be observed. It is discussed that said neuron loss may be due to a damaged microtubule-associated transport system (Johnson and Jenkins, *J Alzheimers Dis* 1996, 1: 38-58; Johnson and Hartigan, *J Alzheimers Dis* 1999, 1: 329-351). The appearance of neurofibrillary tangles and their increasing number correlates well with the clinical severity of AD (Schmitt et al., *Neurology* 2000, 55: 370-376).

AD is a progressive disease that is associated with early deficits in memory formation and ultimately leads to the complete erosion of higher cognitive function. The cognitive disturbances include among other things memory impairment, aphasia, agnosia and the loss of executive functioning. A characteristic feature of the pathogenesis of AD is the selective vulnerability of particular brain regions and subpopulations of nerve cells to the degenerative process. Specifically, the temporal lobe region and the hippocampus are affected early and more severely during the progression of the disease. On the other hand,

neurons within the frontal cortex, occipital cortex, and the cerebellum remain largely intact and are protected from neurodegeneration (Terry et al., *Annals of Neurology* 1981, 10: 184-92).

The age of onset of AD may vary within a range of 50 years, with early-onset AD occurring in people younger than 65 years of age, and late-onset of AD occurring in those older than 65 years. About 10% of all AD cases suffer from early-onset AD, with only 1-2% being familial, inherited cases.

Currently, there is no cure for AD, nor is there an effective treatment to halt the progression of AD or even to diagnose AD ante-mortem with high probability. Several risk factors have been identified that predispose an individual to develop AD, among them most prominently the epsilon 4 allele of the three different existing alleles (epsilon 2, 3, and 4) of the apolipoprotein E gene (ApoE) (Strittmatter et al., *Proc Natl Acad Sci USA* 1993, 90: 1977-81; Roses, *Ann NY Acad Sci* 1998, 855: 738-43). The polymorphic plasmaprotein ApoE plays a role in the intercellular cholesterol and phospholipid transport by binding low-density lipoprotein receptors, and it seems to play a role in neurite growth and regeneration. Studies linking the function of ApoE to AD pathology indicate that ApoE affects amyloid and tau metabolism. Thus, it is discussed to be an important factor for inhibiting axon outgrowth and for neurite and cell loss in AD. Efforts to detect further susceptibility genes and disease-linked polymorphisms, lead to the assumption that specific regions and genes on human chromosomes 10 and 12 may be associated with late-onset AD (Myers et al., *Science* 2000, 290: 2304-5; Bertram et al., *Science* 2000, 290: 2303; Scott et al., *Am J Hum Genet* 2000, 66: 922-32).

Although there are rare examples of early-onset AD which have been attributed to genetic defects in the genes for amyloid precursor protein (APP) on chromosome 21, presenilin-1 on chromosome 14, and presenilin-2 on chromosome 1, the prevalent form of late-onset

sporadic AD is of hitherto unknown etiologic origin. The mutations found to date account for only half of the familial AD cases, which is less than 2% of all AD patients. The late onset and complex pathogenesis of neurodegenerative disorders pose a formidable challenge to the development of therapeutic and diagnostic agents. It is crucial to expand the pool of potential drug targets and diagnostic markers. It is therefore an object of the present invention to provide insight into the pathogenesis of neurological diseases and to provide methods, materials, agents, compositions, and animal models which are suited inter alia for the diagnosis and development of a treatment of these diseases. This object has been solved by the features of the independent claims. The subclaims define preferred embodiments of the present invention.

Voltage-gated ion channels play an important role in the nervous system by generating conducted action potentials. Nowadays, ion-conducting membrane channels for cations (sodium, calcium, potassium) and anions (chloride) are described (Lehmann-Horn et al., *Physiological Reviews* 1999, 79: 1317-1358). Transport of ions across the cell membrane leads to a fast transmission of electrical impulses throughout the cell network. Thereby the channel switches between three functionally distinct states: a resting, an active, and an inactive one. Both, the resting and inactive states are nonconducting, and the channel is closed. As the membrane potential increases from less than -60 mV, the channel starts to open its pore (i.e. activation). Influx of ions (e.g. sodium) leads to a further increase of the membrane potential until an action potential is initiated. By closing the pore within 1 millisecond (i.e. fast inactivation) or within seconds to minutes (i.e. slow inactivation), the channel rapidly returns to an inactivated state. The ion conductance is highly selective and efficient which enables fine tuning of processes such as memory, movement, and cognition (Lehmann-Horn et al., *Physiological Reviews* 1999, 79: 1317-1358). Molecular cloning of voltage-gated ion channels has uncovered a

diversity of subtypes and enhanced the understanding about the underlying structure and function, particularly of sodium channels (Noda et al., *Nature* 1986, 322: 826-828; Schaller et al., *Journal of Neuroscience* 1995, 15: 3231-3242; Isom et al., *Neuron* 1994, 12:1183-1194; Isom et al., *Cell* 1995, 83:443-445). Sodium channels exist as tetramers of four identical homologous domains (DI - DIV), each consisting of six transmembrane helices (S1- S6) which form a group around the central ion-conducting pore. A precise three-dimensional structure is still not available (Catterall et al., *Advances in Neurology* 1999, 79: 441-456). A highly glycosylated α -subunit with approximately 260 kDa and two β -subunits (β 1 with ~36kDa and β 2 with ~33kDa) form a heteromeric complex, whereby the β 1-subunit is noncovalently associated and the β 2-subunit is covalently attached to the α -subunit via a disulfide bridge. A third β -subunit isoform similar to the β 1-subunit, also attached to the α -subunit, has recently been discovered (Morgan et al., *Proceedings National Academy of Science USA* 2000, 97: 2308-2313). The α -subunit appears to be necessary and sufficient for sodium channel functionality. The β -subunit modulates sodium channel function by accelerating activation and inactivation processes by increasing peak current and by altering voltage dependency (Patton et al., *Journal Biological Chemistry* 1994, 269: 17649-17655). β -subunits exhibit an immunoglobulin-like motif with structural similarities to neuronal cell adhesion molecules which may interact with extracellular matrix proteins (Isom et al., *Cell* 1995, 83: 443-445). An important mechanism for modulation of sodium channel properties is the rate of glycosylation and the change in their phosphorylation state. Sodium channels have multiple sites for phosphorylation by protein kinases A and C (PKA and PKC). Phosphorylation of these sites results in slowed inactivation and reduced peak current.

Currently, several different human α -subunit genes have been cloned and found to be organized in four conserved chromosomal segments. They are known to be expressed in mammalian brain and peripheral

tissues, and they show tissue-specific expression with individual cell types expressing different complements of sodium channel subunits. To date, a number of genetic mutations have been identified which affect the function of the above described sodium channels. For example, an underlying cause for generalized epilepsy are mutations in the SCN1A gene (Kearney et al., *Neurosciences* 2001, 2: 307-317). Various periodic paralysis syndromes and hyperexcitability, as found associated with LQT Syndrome, have been linked to mutations in skeletal and cardiac sodium channels (SCN4A, SCN5A) (Lehmann-Horn et al., *Physiological Reviews* 1999, 79: 1317-1358).

Sodium channels are valuable targets for a variety of drugs as local anesthetics, anticonvulsants, antiarrhythmics, for the treatment of neuropathic pain, epilepsy, and stroke. Although a number of toxins, drugs, and inorganic cations are used by the pharmaceutical industry as blockers in central nervous system related disorders, and although a number of inhibitors of voltage-gated ion channels are on the market, the therapeutic potential of currently used drugs is not fully exploited. They are of low potency and relatively non-specific. Thus, it is required to find specific drugs for a selective target known to be associated with a specific clinical condition. To date, there are no reports on a relationship between the voltage-gated sodium channel type 2A (SCN2A) and neurodegenerative disorders such as Alzheimer's disease. Such a link, as disclosed in the present invention, offers new ways, inter alia, for the diagnosis and treatment of these disorders.

The first report about the structure and chromosomal location of sodium channel type 2A (SCN2A) was published in 1992 (denoted as HBA; GenBank Accession No. M94055; X65361; Ahmed et al., *Proc. Natl. Acad. of Sci. USA* 1992, 89: 820-824). A further description of the genomic structure of the SCN2A gene was revealed in 2001 (GenBank Accession No. AF327246; AH010232; GDB ID: 120367; Kasai et al., *Gene* 2001, 264: 113-122). Herein, SCN2A was characterized as a

positional candidate gene for the deafness disorder DFNA16, a form of autosomal dominant non-syndromic hearing loss (ADNSHL). Fine mapping studies clearly define the chromosomal location to the map locus 2q23-q24.3. SCN2A covers approximately 120 kb of genomic DNA, harboring 29 exons (54 bp to 1196 bp in size) which encode for a protein of 2005 amino acids (GenBank Accession No. Q99250). The SCN2A gene is expressed primarily in the central nervous system and in the cochlea. Two alternatively spliced isoforms of SCN2A (exon 6A, exon 6N) were identified, and as a result three mRNA variants were detected, i.e. SCN2A harboring exon 6A, or exon 6N, or none of both. The exon 6A encoding transcript was found to be expressed in human adult brain, and the transcript harboring exon 6N was detected in human fetal brain and lymphocytes. The transcript with deleted exon 6 was found to be expressed in lymphocytes only (Kasai et al., *Gene* 2001, 264: 113-122). In addition to tissue-specific expression of the two alternatively spliced SCN2A isoforms, the SCN2A gene is developmentally regulated. SCN2A type 6A exon is expressed throughout development, with highest levels in rostral brain regions (brainstem, hippocampus, cortex, striatum, midbrain) (Whitaker et al., *Journal of Comparative Neurology* 2000, 422: 123-139; Planells-Cases et al., *Biophysical Journal* 2000, 78: 2878-2891), whereas SCN2A type 6N exon was found to be present only in fetal tissue. The subcellular distribution of SCN2A polypeptides is characterized by location along the axons of neurons, preferentially on unmyelinated projection fibers. This suggests a highly distinct function of the SCN2A channels.

A comparative expression study on the cellular level has been published by Whitaker in 2001 (*Molecular Brain Research* 2001, 88: 37-53). The study compared tissues from normal and from epileptic hippocampus and found SCN2A to be downregulated in pyramidal cells, whereas other sodium channels, such as SCN3A, were upregulated. Recently, several mutations in the SCN2A gene have been identified (Arg1638His; in DIV, S6) (Kasai et al., *Gene* 2001, 264: 113-122), none of which cosegregate

with a pathological phenotype. An animal model for seizure disorders is the so called Q54-mouse. This mouse expresses a transgene with a gain-of-function mutation in domain DII, S4-S5 of the SCN2A gene (Kearney et al., *Neuroscience* 2001, 102: 307-317) resulting in a profound phenotype despite endogenous SCN2A gene expression. A homozygous SCN2A knock-out mouse (deletion of exon 1 of SCN2A gene) shows severe defects and results in mortality around the time of birth (Planells-Cases et al., *Biophysical Journal* 2000, 78: 2878-2891).

The singular forms "a", "an", and "the" as used herein and in the claims include plural reference unless the context dictates otherwise. For example, "a cell" means as well a plurality of cells, and so forth. The term "and/or" as used in the present specification and in the claims implies that the phrases before and after this term are to be considered either as alternatives or in combination. For instance, the wording "determination of a level and/or an activity" means that either only a level, or only an activity, or both a level and an activity are determined. The term "level" as used herein is meant to comprise a gage of, or a measure of the amount of, or a concentration of a transcription product, for instance an mRNA, or a translation product, for instance a protein or polypeptide. The term "activity" as used herein shall be understood as a measure for the ability of a transcription product or a translation product to produce a biological effect or a measure for a level of biologically active molecules. The term "activity" also refers to enzymatic activity. The terms "level" and/or "activity" as used herein further refer to gene expression levels or gene activity. Gene expression can be defined as the utilization of the information contained in a gene by transcription and translation leading to the production of a gene product. "Dysregulation" shall mean an upregulation or downregulation of gene expression. A gene product comprises either RNA or protein and is the result of expression of a gene. The amount of a gene product can be used to measure how active a gene is. The term "gene" as used in

the present specification and in the claims comprises both coding regions (exons) as well as non-coding regions (e.g. non-coding regulatory elements such as promoters or enhancers, introns, leader and trailer sequences). The term "ORF" is an acronym for "open reading frame" and refers to a nucleic acid sequence that does not possess a stop codon in at least one reading frame and therefore can potentially be translated into a sequence of amino acids. "Regulatory elements" shall comprise inducible and non-inducible promoters, enhancers, operators, and other elements that drive and regulate gene expression. The term "fragment" as used herein is meant to comprise e.g. an alternatively spliced, or truncated, or otherwise cleaved transcription product or translation product. The term "derivative" as used herein refers to a mutant, or an RNA-edited, or a chemically modified, or otherwise altered transcription product, or to a mutant, or chemically modified, or otherwise altered translation product. For instance, a "derivative" may be generated by processes such as altered phosphorylation, or glycosylation, or acetylation, or lipidation, or by altered signal peptide cleavage or other types of maturation cleavage. These processes may occur post-translationally. The term "modulator" as used in the present invention and in the claims refers to a molecule capable of changing or altering the level and/or the activity of a gene, or a transcription product of a gene, or a translation product of a gene. Preferably, a "modulator" is capable of changing or altering the biological activity of a transcription product or a translation product of a gene. Said modulation, for instance, may be an increase or a decrease in enzyme activity, a change in binding characteristics, or any other change or alteration in the biological, functional, or immunological properties of said translation product of a gene. The terms "agent", "reagent", or "compound" refer to any substance, chemical, composition or extract that have a positive or negative biological effect on a cell, tissue, body fluid, or within the context of any biological system, or any assay system examined. They can be agonists, antagonists, partial agonists or inverse agonists of a target. Such agents, reagents, or

compounds may be nucleic acids, natural or synthetic peptides or protein complexes, or fusion proteins. They may also be antibodies, organic or anorganic molecules or compositions, small molecules, drugs and any combinations of any of said agents above. They may be used for testing, for diagnostic or for therapeutic purposes. The terms "oligonucleotide primer" or "primer" refer to short nucleic acid sequences which can anneal to a given target polynucleotide by hybridization of the complementary base pairs and can be extended by a polymerase. They may be chosen to be specific to a particular sequence or they may be randomly selected, e.g. they will prime all possible sequences in a mix. The length of primers used herein may vary from 10 nucleotides to 80 nucleotides. "Probes" are short nucleic acid sequences of the nucleic acid sequences described and disclosed herein or sequences complementary therewith. They may comprise full length sequences, or fragments, derivatives, isoforms, or variants of a given sequence. The identification of hybridization complexes between a "probe" and an assayed sample allows the detection of the presence of other similar sequences within that sample. As used herein, "homolog or homology" is a term used in the art to describe the relatedness of a nucleotide or peptide sequence to another nucleotide or peptide sequence, which is determined by the degree of identity and/or similarity between said sequences compared. The term "variant" as used herein refers to any polypeptide or protein, in reference to polypeptides and proteins disclosed in the present invention, in which one or more amino acids are added and/or substituted and/or deleted and/or inserted at the N-terminus, and/or the C-terminus, and/or within the native amino acid sequences of the native polypeptides or proteins of the present invention. Furthermore, the term "variant" shall include any shorter or longer version of a polypeptide or protein. "Variants" shall also comprise a sequence that has at least about 80% sequence identity, more preferably at least about 90% sequence identity, and most preferably at least about 95% sequence identity with the amino acid sequences of the voltage-gated sodium channel protein SCN2A.

"Variants" of a protein molecule include, for example, proteins with conservative amino acid substitutions in highly conservative regions. "Proteins and polypeptides" of the present invention include variants, fragments and chemical derivatives of the protein comprising the amino acid sequences of SCN2A. They can include proteins and polypeptides which can be isolated from nature or be produced by recombinant and/or synthetic means. Native proteins or polypeptides refer to naturally-occurring truncated or secreted forms, naturally occurring variant forms (e.g. splice-variants) and naturally occurring allelic variants. The term "isolated" as used herein is considered to refer to molecules that are removed from their natural environment, i.e. isolated from a cell or from a living organism in which they normally occur, and that are separated or essentially purified from the coexisting components with which they are found to be associated in nature. This notion further means that the sequences encoding such molecules can be linked by the hand of man to polynucleotides, to which they are not linked in their natural state, and that such molecules can be produced by recombinant and/or synthetic means. Even if for said purposes those sequences may be introduced into living or non-living organisms by methods known to those skilled in the art, and even if those sequences are still present in said organisms, they are still considered to be isolated. In the present invention, the terms "risk", "susceptibility", and "predisposition" are tantamount and are used with respect to the probability of developing a neurodegenerative disease, preferably Alzheimer's disease.

The term 'AD' shall mean Alzheimer's disease. "AD-type neuropathology" as used herein refers to neuropathological, neurophysiological, histopathological and clinical hallmarks as described in the instant invention and as commonly known from state-of-the-art literature (see: Iqbal, Swaab, Winblad and Wisniewski, *Alzheimer's Disease and Related Disorders (Etiology, Pathogenesis and Therapeutics)*, Wiley & Sons, New York, Weinheim, Toronto, 1999; Scinto and Daffner, *Early Diagnosis of Alzheimer's Disease*, Humana

Press, Totowa, New Jersey, 2000; Mayeux and Christen, *Epidemiology of Alzheimer's Disease: From Gene to Prevention*, Springer Press, Berlin, Heidelberg, New York, 1999; Younkin, Tanzi and Christen, *Presenilins and Alzheimer's Disease*, Springer Press, Berlin, Heidelberg, New York, 1998).

Neurodegenerative diseases or disorders according to the present invention comprise Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Pick's disease, fronto-temporal dementia, progressive nuclear palsy, corticobasal degeneration, cerebro-vascular dementia, multiple system atrophy, argyrophilic grain dementia and other tauopathies, and mild-cognitive impairment. Further conditions involving neurodegenerative processes are, for instance, age-related macular degeneration, narcolepsy, motor neuron diseases, prion diseases, traumatic nerve injury and repair, and multiple sclerosis.

In one aspect, the invention features a method of diagnosing or prognosticating a neurodegenerative disease in a subject, or determining whether a subject is at increased risk of developing said disease. The method comprises: determining a level, or an activity, or both said level and said activity of (i) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (ii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (iii) a fragment, or derivative, or variant of said transcription or translation product in a sample from said subject and comparing said level, and/or said activity to a reference value representing a known disease or health status, thereby diagnosing or prognosticating said neurodegenerative disease in said subject, or determining whether said subject is at increased risk of developing said neurodegenerative disease.

The invention also relates to the construction and the use of primers and probes which are unique to the nucleic acid sequences, or

fragments or variants thereof, as disclosed in the present invention. The oligonucleotide primers and/or probes can be labeled specifically with fluorescent, bioluminescent, magnetic, or radioactive substances. The invention further relates to the detection and the production of said nucleic acid sequences, or fragments and variants thereof, using said specific oligonucleotide primers in appropriate combinations. PCR-analysis, a method well known to those skilled in the art, can be performed with said primer combinations to amplify said gene specific nucleic acid sequences from a sample containing nucleic acids. Such sample may be derived either from healthy or diseased subjects. Whether an amplification results in a specific nucleic acid product or not, and whether a fragment of different length can be obtained or not, may be indicative for a neurodegenerative disease, in particular Alzheimer's disease. Thus, the invention provides nucleic acid sequences, oligonucleotide primers, and probes of at least 10 bases in length up to the entire coding and gene sequences, useful for the detection of gene mutations and single nucleotide polymorphisms in a given sample comprising nucleic acid sequences to be examined, which may be associated with neurodegenerative diseases, in particular Alzheimers disease. This feature has utility for developing rapid DNA-based diagnostic tests, preferably also in the format of a kit.

In a further aspect, the invention features a method of monitoring the progression of a neurodegenerative disease in a subject. A level, or an activity, or both said level and said activity, of (i) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (ii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (iii) a fragment, or derivative, or variant of said transcription or translation product in a sample from said subject is determined. Said level and/or said activity is compared to a reference value representing a known disease or health status. Thereby, the progression of said neurodegenerative disease in said subject is monitored.

In still a further aspect, the invention features a method of evaluating a treatment for a neurodegenerative disease, comprising determining a level, or an activity, or both said level and said activity of (i) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (ii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (iii) a fragment, or derivative, or variant of said transcription or translation product in a sample obtained from a subject being treated for said disease. Said level, or said activity, or both said level and said activity are compared to a reference value representing a known disease or health status, thereby evaluating the treatment for said neurodegenerative disease.

In a preferred embodiment of the herein claimed methods, kits, recombinant animals, molecules, assays, and uses of the instant invention, said gene coding for the voltage-gated ion channel protein is the gene coding for the human α -subunit voltage-gated sodium channel type II (SCN2A), also termed voltage-gated sodium channel type II alpha or voltage-gated ion channel SCN2A (SEQ ID NO. 2, constructed from Genbank accession numbers: AF327224 - AF327246).

In a further preferred embodiment of the herein claimed methods, kits, recombinant animals, molecules, assays, and uses of the instant invention, said neurodegenerative disease or disorder is Alzheimer's disease, and said subjects suffer from Alzheimer's disease.

The present invention discloses the detection and differential expression and regulation of the SCN2A gene in specific brain regions of Alzheimer's disease patients. Consequently, the SCN2A gene and its corresponding transcription and/or translation products may have a causative role in the regional selective neuronal degeneration typically observed in Alzheimer's disease. Alternatively, SCN2A may confer a neuroprotective function to the remaining surviving nerve cells. Based

on these disclosures, the present invention has utility for the diagnostic evaluation and prognosis as well as for the identification of a predisposition to a neurodegenerative disease, in particular Alzheimer's disease. Furthermore, the present invention provides methods for the diagnostic monitoring of patients undergoing treatment for such a disease.

It is preferred that the sample to be analyzed and determined is selected from the group comprising brain tissue or other body cells. The sample can also comprise cerebrospinal fluid or other body fluids including saliva, urine, serum plasma, or mucus. Preferably, the methods of diagnosis, prognosis, monitoring the progression or evaluating a treatment for a neurodegenerative disease, according to the instant invention, can be practiced *ex corpore*, and such methods preferably relate to samples, for instance, body fluids or cells, removed, collected, or isolated from a subject or patient.

In further preferred embodiments, said reference value is that of a level, or an activity, or both said level and said activity of (i) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (ii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (iii) a fragment, or derivative, or variant of said transcription or translation product in a sample from a subject not suffering from said neurodegenerative disease.

In preferred embodiments, an alteration in the level and/or activity of a transcription product of the gene coding for SCN2A and/or a translation product of the gene coding for SCN2A in a sample cell, or tissue, or body fluid from said subject relative to a reference value representing a known health status indicates a diagnosis, or prognosis, or increased risk of becoming diseased with a neurodegenerative disease, particularly AD.

In preferred embodiments, measurement of the level of transcription products of the gene coding for the voltage-gated ion channel SCN2A is performed in a sample from a subject using a quantitative PCR-analysis with primer combinations to amplify said gene specific sequences from cDNA obtained by reverse transcription of RNA extracted from a sample of a subject. A Northern blot with probes specific for said gene can also be applied. It might further be preferred to measure transcription products by means of chip-based microarray technologies. These techniques are known to those of ordinary skill in the art (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001; Schena M., *Microarray Biochip Technology*, Eaton Publishing, Natick, MA, 2000). An example of an immunoassay is the detection and measurement of enzyme activity as disclosed and described in the patent application WO 02/14543.

Furthermore, a level and/or activity of a translation product of the gene coding for the voltage-gated ion channel SCN2A and/or fragment, or derivative, or variant of said translation product, and/or level of activity of said translation product can be detected using an immunoassay, an activity assay, and/or binding assay. These assays can measure the amount of binding between said protein molecule and an anti-protein antibody by the use of enzymatic, chromodynamic, radioactive, magnetic, or luminescent labels which are attached to either the anti-protein antibody or a secondary antibody which binds the anti-protein antibody. In addition, other high affinity ligands may be used. Immunoassays which can be used include e.g. ELISAs, Western blots and other techniques known to those of ordinary skill in the art (see Harlow and Lane, *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999 and Edwards R, *Immunodiagnosics: A Practical Approach*, Oxford University Press, Oxford; England, 1999). All these detection techniques may also

be employed in the format of microarrays, protein-arrays, antibody microarrays, tissue microarrays, electronic biochip or protein-chip based technologies (see Schena M., *Microarray Biochip Technology*, Eaton Publishing, Natick, MA, 2000).

In a preferred embodiment, the level, or the activity, or both said level and said activity of (i) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (ii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or of (iii) a fragment, or derivative, or variant of said transcription or translation product in a series of samples taken from said subject over a period of time is compared, in order to monitor the progression of said disease. In further preferred embodiments, said subject receives a treatment prior to one or more of said sample gatherings. In yet another preferred embodiment, said level and/or activity is determined before and after said treatment of said subject.

In another aspect, the invention features a kit for diagnosing or prognosticating neurodegenerative diseases, in particular Alzheimer's disease, in a subject, or determining the propensity or predisposition of a subject to develop a neurodegenerative disease, in particular Alzheimer's disease, said kit comprising:

(a) at least one reagent which is selected from the group consisting of (i) reagents that selectively detect a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and (ii) reagents that selectively detect a translation product of the gene coding for the voltage-gated ion channel SCN2A; and

(b) an instruction for diagnosing, or prognosticating a neurodegenerative disease, in particular Alzheimer's disease, or determining the propensity or predisposition of a subject to develop such a disease by

- detecting a level, or an activity, or both said level and said activity, of said transcription product and/or said translation

product of the gene coding for the voltage-gated ion channel SCN2A, in a sample from said subject; and

- diagnosing or prognosticating a neurodegenerative disease, in particular Alzheimer's disease, or determining the propensity or predisposition of said subject to develop such a disease,

wherein a varied level, or activity, or both said level and said activity, of said transcription product and/or said translation product compared to a reference value representing a known health status; or a level, or activity, or both said level and said activity, of said transcription product and/or said translation product similar or equal to a reference value representing a known disease status, indicates a diagnosis or prognosis of a neurodegenerative disease, in particular Alzheimer's disease, or an increased propensity or predisposition of developing such a disease. The kit, according to the present invention, may be particularly useful for the identification of individuals that are at risk of developing a neurodegenerative disease, in particular Alzheimer's disease. Consequently, the kit, according to the invention, may serve as a means for targeting identified individuals for early preventive measures or therapeutic intervention prior to disease onset, before irreversible damage in the course of the disease has been inflicted. Furthermore, in preferred embodiments, the kit featured in the invention is useful for monitoring a progression of a neurodegenerative disease, in particular Alzheimer's disease, in a subject, as well as monitoring success or failure of therapeutic treatment for such a disease of said subject.

In another aspect, the invention features a method of treating or preventing a neurodegenerative disease, in particular Alzheimer's disease, in a subject comprising the administration to said subject in a therapeutically or prophylactically effective amount of an agent or agents which directly or indirectly affect a level, or an activity, or both said level and said activity, of (i) the gene coding for the voltage-gated ion channel SCN2A, and/or (ii) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iii) a

translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iv) a fragment, or derivative, or variant of (i) to (iii). Said agent may comprise a small molecule, or it may also comprise a peptide, an oligopeptide, or a polypeptide. Said peptide, oligopeptide, or polypeptide may comprise an amino acid sequence of a translation product of the gene coding for SCN2A protein, or a fragment, or derivative, or a variant thereof. An agent for treating or preventing a neurodegenerative disease, in particular AD, according to the instant invention, may also consist of a nucleotide, an oligonucleotide, or a polynucleotide. Said oligonucleotide or polynucleotide may comprise a nucleotide sequence of the gene coding for SCN2A protein, either in sense orientation or in antisense orientation.

In preferred embodiments, the method comprises the application of per se known methods of gene therapy and/or antisense nucleic acid technology to administer said agent or agents. In general, gene therapy includes several approaches: molecular replacement of a mutated gene, addition of a new gene resulting in the synthesis of a therapeutic protein, and modulation of endogenous cellular gene expression by recombinant expression methods or by drugs. Gene-transfer techniques are described in detail (see e.g. Behr, *Acc Chem Res* 1993, 26: 274-278 and Mulligan, *Science* 1993, 260: 926-931) and include direct gene-transfer techniques such as mechanical microinjection of DNA into a cell as well as indirect techniques employing biological vectors (like recombinant viruses, especially retroviruses) or model liposomes, or techniques based on transfection with DNA coprecipitation with polycations, cell membrane perturbation by chemical (solvents, detergents, polymers, enzymes) or physical means (mechanic, osmotic, thermic, electric shocks). The postnatal gene transfer into the central nervous system has been described in detail (see e.g. Wolff, *Curr Opin Neurobiol* 1993, 3: 743-748).

In particular, the invention features a method of treating or preventing a neurodegenerative disease by means of antisense nucleic acid therapy, i.e. the down-regulation of an inappropriately expressed or defective gene by the introduction of antisense nucleic acids or derivatives thereof into certain critical cells (see e.g. Gillespie, *DN&P* 1992, 5: 389-395; Agrawal and Akhtar, *Trends Biotechnol* 1995, 13: 197-199; Crooke, *Biotechnology* 1992, 10: 882-6; the contents of which are incorporated herein by reference). Apart from hybridization strategies, the application of ribozymes, i.e. RNA molecules that act as enzymes, destroying RNA that carries the message of disease has also been described (see e.g. Barinaga, *Science* 1993, 262: 1512-1514). In preferred embodiments, the subject to be treated is a human, and therapeutic antisense nucleic acids or derivatives thereof are directed against transcripts of the gene coding for the human voltage-gated ion channel SCN2A. It is preferred that cells of the central nervous system, preferably the brain, of a subject are treated in such a way. Cell penetration can be performed by known strategies such as coupling of antisense nucleic acids and derivatives thereof to carrier particles, or the above described techniques. Strategies for administering targeted therapeutic oligodeoxynucleotides are known to those of skill in the art (see e.g. Wickstrom, *Trends Biotechnol* 1992, 10: 281-287). In some cases, delivery can be performed by mere topical application. Further approaches are directed to intracellular expression of antisense RNA. In this strategy, cells are transformed *ex vivo* with a recombinant gene that directs the synthesis of an RNA that is complementary to a region of target nucleic acid. Therapeutical use of intracellularly expressed antisense RNA is procedurally similar to gene therapy. A recently developed method of regulating the intracellular expression of genes by the use of double-stranded RNA, known variously as RNA interference (RNAi), can be another effective approach for nucleic acid therapy (Hannon, *Nature* 2002, 418: 244-251).

In further preferred embodiments, the method comprises grafting donor cells into the central nervous system, preferably the brain, of said subject, or donor cells preferably treated so as to minimize or reduce graft rejection, wherein said donor cells are genetically modified by insertion of at least one transgene encoding said agent or agents. Said transgene might be carried by a viral vector, in particular a retroviral vector. The transgene can be inserted into the donor cells by a nonviral physical transfection of DNA encoding a transgene, in particular by microinjection. Insertion of the transgene can also be performed by electroporation, chemically mediated transfection, in particular calcium phosphate transfection or liposomal mediated transfection (see McClland and Pardee, *Expression Genetics: Accelerated and High-Throughput Methods*, Eaton Publishing, Natick, MA, 1999).

In preferred embodiments, said agent for treating and preventing a neurodegenerative disease, in particular AD, is a therapeutic protein which can be administered to said subject, preferably a human, by a process comprising introducing subject cells into said subject, said subject cells having been treated *in vitro* to insert a DNA segment encoding said therapeutic protein, said subject cells expressing *in vivo* in said subject a therapeutically effective amount of said therapeutic protein. Said DNA segment can be inserted into said cells *in vitro* by a viral vector, in particular a retroviral vector.

Methods of treatment, according to the present invention, comprise the application of therapeutic cloning, transplantation, and stem cell therapy using embryonic stem cells or embryonic germ cells and neuronal adult stem cells, combined with any of the previously described cell- and gene therapeutic methods. Stem cells may be totipotent or pluripotent. They may also be organ-specific. Strategies for repairing diseased and/or damaged brain cells or tissue comprise (i) taking donor cells from an adult tissue. Nuclei of those cells are transplanted into unfertilized egg cells from which the genetic material

has been removed. Embryonic stem cells are isolated from the blastocyst stage of the cells which underwent somatic cell nuclear transfer. Use of differentiation factors then leads to a directed development of the stem cells to specialized cell types, preferably neuronal cells (Lanza et al., *Nature Medicine* 1999, 9: 975-977), or (ii) purifying adult stem cells, isolated from the central nervous system, or from bone marrow (mesenchymal stem cells), for in vitro expansion and subsequent grafting and transplantation, or (iii) directly inducing endogenous neural stem cells to proliferate, migrate, and differentiate into functional neurons (Peterson DA, *Curr. Opin. Pharmacol.* 2002, 2: 34-42). Adult neural stem cells are of great potential for repairing damaged or diseased brain tissues, as the germinal centers of the adult brain are free of neuronal damage or dysfunction (Colman A, *Drug Discovery World* 2001, 7: 66-71).

In preferred embodiments, the subject for treatment or prevention, according to the present invention, can be a human, an experimental animal, e.g. a mouse or a rat, a domestic animal, or a non-human primate. The experimental animal can be an animal model for a neurodegenerative disorder, e.g. a transgenic mouse and/or a knock-out mouse with an Alzheimer's-type neuropathology.

In a further aspect, the invention features a modulator of an activity, or a level, or both said activity and said level of at least one substance which is selected from the group consisting of (i) the gene coding for the voltage-gated ion channel SCN2A, and/or (ii) a transcription product of the gene coding for the voltage-gated ion channel SCN2A and/or (iii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iv) a fragment, or derivative, or variant of (i) to (iii).

In an additional aspect, the invention features a pharmaceutical composition comprising said modulator and preferably a pharmaceutical

carrier. Said carrier refers to a diluent, adjuvant, excipient, or vehicle with which the modulator is administered.

In a further aspect, the invention features a modulator of an activity, or a level, or both said activity and said level of at least one substance which is selected from the group consisting of (i) the gene coding for the voltage-gated ion channel SCN2A, and/or (ii) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iv) a fragment, or derivative, or variant of (i) to (iii) for use in a pharmaceutical composition.

In another aspect, the invention provides for the use of a modulator of an activity, or a level, or both said activity and said level of at least one substance which is selected from the group consisting of (i) the gene coding for the voltage-gated ion channel SCN2A, and/or (ii) a transcription product of the gene coding for the voltage-gated ion channel SCN2A and/or (iii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iv) a fragment, or derivative, or variant of (i) to (iii) for a preparation of a medicament for treating or preventing a neurodegenerative disease, in particular Alzheimer's disease.

In one aspect, the present invention also provides a kit comprising one or more containers filled with a therapeutically or prophylactically effective amount of said pharmaceutical composition.

In a further aspect, the invention features a recombinant, non-human animal comprising a non-native gene sequence coding for the voltage-gated ion channel SCN2A, or a fragment, or a derivative, or variant thereof. The generation of said recombinant, non-human animal comprises (i) providing a gene targeting construct containing said gene sequence and a selectable marker sequence, and (ii) introducing said

targeting construct into a stem cell of a non-human animal, and (iii) introducing said non-human animal stem cell into a non-human embryo, and (iv) transplanting said embryo into a pseudopregnant non-human animal, and (v) allowing said embryo to develop to term, and (vi) identifying a genetically altered non-human animal whose genome comprises a modification of said gene sequence in both alleles, and (vii) breeding the genetically altered non-human animal of step (vi) to obtain a genetically altered non-human animal whose genome comprises a modification of said endogenous gene, wherein said gene is mis-expressed, or under-expressed, or over-expressed, and wherein said disruption or alteration results in said non-human animal exhibiting a predisposition to developing symptoms of neuropathology similar to a neurodegenerative disease, in particular Alzheimer's disease. Strategies and techniques for the generation and construction of such an animal are known to those of ordinary skill in the art (see e.g. Capecchi, *Science* 1989, 244: 1288-1292 and Hogan et al., 1994, *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York and Jackson and Abbott, *Mouse Genetics and Transgenics: A Practical Approach*, Oxford University Press, Oxford, England, 1999). It is preferred to make use of such a recombinant non-human animal as an animal model for investigating neurodegenerative diseases, in particular Alzheimer's disease. Such an animal may be useful for screening, testing and validating compounds, agents and modulators in the development of diagnostics and therapeutics to treat neurodegenerative diseases, in particular Alzheimer's disease.

In another aspect, the invention features an assay for screening for a modulator of neurodegenerative diseases, in particular Alzheimer's disease, or related diseases and disorders of one or more substances selected from the group consisting of (i) the gene coding for the voltage-gated ion channel SCN2A, and/or (ii) a transcription product of

the gene coding for the voltage-gated ion channel SCN2A, and/or (iii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iv) a fragment, or derivative, or variant of (i) to (iii). This screening method comprises (a) contacting a cell with a test compound, and (b) measuring the activity, or the level, or both the activity and the level of one or more substances recited in (i) to (iv), and (c) measuring the activity, or the level, or both the activity and the level of said substances in a control cell not contacted with said test compound, and (d) comparing the levels of the substance in the cells of step (b) and (c), wherein an alteration in the activity and/or level of said substances in the contacted cells indicates that the test compound is a modulator of said diseases and disorders.

In one further aspect, the invention features a screening assay for a modulator of neurodegenerative diseases, in particular Alzheimer's disease, or related diseases and disorders of one or more substances selected from the group consisting of (i) the gene coding for the voltage-gated ion channel SCN2A, and/or (ii) a transcription product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iii) a translation product of the gene coding for the voltage-gated ion channel SCN2A, and/or (iv) a fragment, or derivative, or variant of (i) to (iii), comprising (a) administering a test compound to a test animal which is predisposed to developing or has already developed symptoms of a neurodegenerative disease or related diseases or disorders, and (b) measuring the activity and/or level of one or more substances recited in (i) to (iv), and (c) measuring the activity and/or level of said substances in a matched control animal which is equally predisposed to developing or has already developed said symptoms and to which animal no such test compound has been administered, and (d) comparing the activity and/or level of the substance in the animals of step (b) and (c), wherein an alteration in the activity and/or level of substances in the test animal indicates that the test compound is a modulator of said diseases and disorders.

In a preferred embodiment, said test animal and/or said control animal is a recombinant, non-human animal which expresses the gene coding for the voltage-gated ion channel SCN2A, or a fragment, or derivative, or variant thereof, under the control of a transcriptional regulatory element which is not the native SCN2A voltage-gated ion channel gene transcriptional control regulatory element.

In another embodiment, the present invention provides a method for producing a medicament comprising the steps of (i) identifying a modulator of neurodegenerative diseases by a method of the aforementioned screening assays and (ii) admixing the modulator with a pharmaceutical carrier. However, said modulator may also be identifiable by other types of screening assays.

In another aspect, the present invention provides for an assay for testing a compound, preferably for screening a plurality of compounds, for inhibition of binding between a ligand and a translation product of the gene coding for the voltage-gated ion channel SCN2A, or a fragment, or derivative, or variant thereof. Said screening assay comprises the steps of (i) adding a liquid suspension of said voltage-gated ion channel SCN2A translation product, or a fragment, or derivative, or variant thereof, to a plurality of containers, and (ii) adding a compound or a plurality of compounds to be screened for said inhibition to said plurality of containers, and (iii) adding fluorescently labelled ligand to said containers, and (iv) incubating said voltage-gated ion channel SCN2A translation product, or said fragment, or derivative, or variant thereof, and said compound or plurality of compounds, and said fluorescently labelled ligand, and (v) measuring the amounts of fluorescence associated with said voltage-gated ion channel SCN2A translation product, or with said fragment, or derivative, or variant thereof, and (vi) determining the degree of inhibition by one or more of said compounds of binding of said ligand to said voltage-gated ion

channel SCN2A translation product, or said fragment, or derivative, or variant thereof. Instead of utilizing a fluorescently labelled ligand, it might in some aspects be preferred to use any other detectable label known to the person skilled in the art, e.g. radioactive labels, and detect it accordingly. Said method may be useful for the identification of novel compounds as well as for evaluating compounds which have been improved or otherwise optimized in their ability to inhibit the binding of a ligand to a gene product of the gene coding for the voltage-gated ion channel SCN2A, or a fragment, or derivative, or variant thereof. One example of a fluorescent binding assay, in this case based on the use of carrier particles, is disclosed and described in patent application WO 00/52451. A further example is the competitive assay method as described in patent WO 02/01226. Preferred signal detection methods for screening assays of the instant invention are described in the following patent applications: WO 96/13744, WO 98/16814, WO 98/23942, WO 99/17086, WO 99/34195, WO 00/66985, WO 01/59436, WO 01/59416.

In one further embodiment, the present invention provides a method for producing a medicament comprising the steps of (i) identifying a compound as an inhibitor of binding between a ligand and a gene product of the gene coding for the voltage-gated ion channel SCN2A by the aforementioned inhibitory binding assay and (ii) admixing the compound with a pharmaceutical carrier. However, said compound may also be identifiable by other types of screening assays.

In another aspect, the invention features an assay for testing a compound, preferably for screening a plurality of compounds to determine the degree of binding of said compounds to a translation product of the gene coding for the voltage-gated ion channel SCN2A, or to a fragment, or derivative, or variant thereof. Said screening assay comprises (i) adding a liquid suspension of said voltage-gated ion channel SCN2A translation product, or a fragment, or derivative, or

variant thereof, to a plurality of containers, and (ii) adding a fluorescently labelled compound or a plurality of fluorescently labelled compounds to be screened for said binding to said plurality of containers, and (iii) incubating said voltage-gated ion channel SCN2A translation product, or said fragment, or derivative, or variant thereof, and said fluorescently labelled compound or fluorescently labelled compounds, and (iv) measuring the amounts of fluorescence associated with said voltage-gated ion channel SCN2A translation product, or with said fragment, or derivative, or variant thereof, and (v) determining the degree of binding by one or more of said compounds to said voltage-gated ion channel SCN2A translation product, or said fragment, or derivative, or variant thereof. In this type of assay it might be preferred to use a fluorescent label. However, any other type of detectable label might also be employed. Said method may be useful for the identification of novel compounds as well as for evaluating compounds which have been improved or otherwise optimized in their ability to bind to a voltage-gated ion channel SCN2A translation product, or fragment, or derivative, or variant thereof.

In one further embodiment, the present invention provides a method for producing a medicament comprising the steps of (i) identifying a compound as a binder to a gene product of the gene coding for the voltage-gated ion channel SCN2A by the aforementioned binding assays and (ii) admixing the compound with a pharmaceutical carrier. However, said compound may also be identifiable by other types of screening assays.

In another embodiment, the present invention provides for a medicament obtainable by any of the methods according to the herein claimed screening assays. In one further embodiment, the instant invention provides for a medicament obtained by any of the methods according to the herein claimed screening assays.

The present invention features a protein molecule shown in SEQ ID NO: 3, or a fragment, or derivative, or variant thereof, for use as a diagnostic target for detecting a neurodegenerative disease, preferably Alzheimer's disease.

The present invention further features a protein molecule shown in SEQ ID NO: 3, or a fragment, or derivative, or variant thereof, for use as a screening target for reagents or compounds preventing, or treating, or ameliorating a neurodegenerative disease, preferably Alzheimer's disease.

The present invention features an antibody which is specifically immunoreactive with an immunogen, wherein said immunogen is a translation product of the gene coding for the voltage-gated ion channel SCN2A or a fragment, or variant, or derivative thereof. The immunogen may comprise immunogenic or antigenic epitopes or portions of a translation product of said gene, wherein said immunogenic or antigenic portion of a translation product is a polypeptide, and wherein said polypeptide elicits an antibody response in an animal, and wherein said polypeptide is immunospecifically bound by said antibody. Methods for generating antibodies are well known in the art (see Harlow et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988). The term "antibody", as employed in the present invention, encompasses all forms of antibodies known in the art, such as polyclonal, monoclonal, chimeric, recombinatorial, anti-idiotypic, humanized, or single chain antibodies, as well as fragments thereof (see Dubel and Breitling, *Recombinant Antibodies*, Wiley-Liss, New York, NY, 1999). Antibodies of the present invention are useful, for instance, in a variety of diagnostic and therapeutic methods, based on state-in-the-art techniques (see Harlow and Lane, *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999 and Edwards R., *Immunodiagnosics: A Practical Approach*, Oxford University Press, Oxford, England, 1999).

such as enzyme-immuno assays (e.g. enzyme-linked immunosorbent assay, ELISA), radioimmuno assays, chemoluminescence-immuno assays, Western-blot, immunoprecipitation and antibody microarrays. These methods involve the detection of translation products of the SCN2A gene.

In a preferred embodiment of the present invention, said antibodies can be used for detecting the pathological state of a cell in a sample from a subject, comprising immunocytochemical staining of said cell with said antibody, wherein an altered degree of staining, or an altered staining pattern in said cell compared to a cell representing a known health status indicates a pathological state of said cell. Preferably, the pathological state relates to a neurodegenerative disease, in particular to Alzheimer's disease. Immuno-cytochemical staining of a cell can be carried out by a number of different experimental methods well known in the art. It might be preferred, however, to apply an automated method for the detection of antibody binding, wherein the determination of the degree of staining of a cell, or the determination of the cellular or subcellular staining pattern of a cell, or the topological distribution of an antigen on the cell surface or among organelles and other subcellular structures within the cell, are carried out according to the method described in US patent 6150173.

Other features and advantages of the invention will be apparent from the following description of figures and examples which are illustrative only and not intended to limit the remainder of the disclosure in any way.

Figure 1 depicts the brain regions with selective vulnerability to neuronal loss and degeneration in Alzheimer's disease. Primarily, neurons within the inferior temporal lobe, the entorhinal cortex, the hippocampus, and the amygdala are subject to degenerative processes in Alzheimer's disease (Terry et al., *Annals of Neurology* 1981, 10:184-

192). These brain regions are mostly involved in the processing of learning and memory functions. In contrast, neurons within the frontal cortex, the occipital cortex, and the cerebellum remain largely intact and preserved from neurodegenerative processes in Alzheimer's disease. Brain tissues from the frontal cortex (F), the temporal cortex (T), and the hippocampus (H) of Alzheimer's disease patients and healthy, age-matched control individuals were used for the herein disclosed examples. For illustrative purposes, the image of a normal healthy brain was taken from a publication by Strange (*Brain Biochemistry and Brain Disorders*, Oxford University Press, Oxford, 1992, p.4).

Figure 2 discloses the initial identification of the differential expression of the gene coding for the voltage-gated ion channel SCN2A in a suppressive subtractive hybridization screen. The figure shows a clipping of a large-scale dot blot hybridization experiment. Individual cDNA clones from a temporally subtracted library were arrayed onto a nylon membrane and hybridized with probes enriched for genes expressed in the frontal cortex (F) and the temporal cortex (T) of an Alzheimer's disease patient. Ia) clone T16-F11; Ib) clone T16-G11; Ic) clone T16-H11; SCN2A ; IIa) clone T16-F12; IIb) clone T16-G12; IIc) clone T16-H12. Note the significantly stronger intensity of the hybridization signal for SCN2A in panel (F) (see arrow head) as compared to the signal in panel (T).

Figures 3 and 4 illustrate the verification of the differential expression of the human SCN2A gene in AD brain tissues by quantitative RT-PCR analysis. Quantification of RT-PCR products from RNA samples collected from the frontal cortex (F), the temporal cortex (T), and the hippocampus (H) of Alzheimer's disease patients (Figures 3a and 4a) and of healthy, age-matched control individuals (Figures 3b and 4b) was performed by the LightCycler rapid thermal cycling technique. The data were normalized to the combined average values of a set of standard

genes which showed no significant differences in their gene expression levels. Said set of standard genes consisted of genes for the ribosomal protein S9, the transferrin receptor, GAPDH, cyclophilin B, and beta-actin. The figure depicts the kinetics of amplification by plotting the cycle number against the amount of amplified material as measured by its fluorescence. Note that the amplification kinetics of SCN2A cDNA from both, the frontal and temporal cortices of a normal control individual, and from the frontal cortex and hippocampus of a normal control individual, respectively, during the exponential phase of the reaction are juxtaposed (Figures 3b and 4b, arrowheads), whereas in Alzheimer's disease (Figures 3a and 4a, arrowheads) there is a significant separation of the corresponding curves, indicating a differential expression of the SCN2A gene in the respective analyzed brain regions.

Figure 5 depicts SEQ ID NO: 1, the nucleotide sequence of the 272 bp SCN2A cDNA fragment, identified and obtained by suppressive subtractive hybridization cloning (sequence in 5' to 3' direction).

Figure 6 charts the schematic alignment of SEQ ID NO: 1, the SCN2A cDNA fragment, with the nucleotide sequence of the human α -subunit of the voltage-gated sodium channel type II (SCN2A) (constructed from GenBank accession numbers AF327224 - AF327246). The open rectangle represents the SCN2A open reading frame, thin bars represent the 3' and 5' untranslated regions (UTR), respectively. The SCN2A cDNA fragment is located within the 3'UTR and is identical to a part of exon 27 of the 8292 bp full-length SCN2A cDNA.

Figure 7 outlines the sequence alignment of SEQ ID NO: 1, the 272 bp SCN2A cDNA fragment, to the nucleotide sequence of the human voltage-gated sodium channel type II A cDNA (SCN2A), SEQ ID NO: 2 (constructed from GenBank accession numbers AF327224 - AF327246).

Figure 8 shows SEQ ID NO: 2, the nucleotide sequence of the human SCN2A cDNA, comprising 8292 nucleotides, constructed from GenBank accession numbers AF327224 - AF327246 according to the instructions in GenBank accession number AF327246.

Figure 9 discloses SEQ ID NO: 3, the amino acid sequence of the SCN2A protein (GenBank accession number Q99250). The full-length human SCN2A protein comprises 2005 amino acids.

Figure 10 depicts human cerebral cortex labeled with anti-SCN2A mouse monoclonal antibodies (green signal). Immunoreactivity of the voltage-gated sodium channel SCN2A was detected in the pre-central cortex (CT) but not in the white matter (WM) (Figure 10a, low magnification). The cortex showed punctate immunoreactive signals that decorated neuronal cell processes, whereas most of the neuronal cell bodies were immuno-negative (Figure 10b, high magnification). In contrast, a positively stained cell body is indicated (see arrow). Blue signals indicate nuclei stained with DAPI.

Table 1 lists the SCN2A gene expression levels in the frontal cortex relative to the temporal cortex in seven Alzheimer's disease patients, herein identified by internal reference numbers P010, P011, P012, P014, P016, P017, P019 (0.97 to 3.16 fold) and five healthy, age-matched control individuals, herein identified by internal reference numbers C005, C008, C011, C012, C014 (0.52 to 1.07 fold). The values shown are reciprocal values according to the formula described herein (see below).

Table 2 lists the SCN2A gene expression levels in the frontal cortex relative to the hippocampus in six Alzheimer's disease patients, herein identified by internal reference numbers P010, P011, P012, P014, P016, P019 (0.82 to 6.68 fold) and three healthy, age-matched control individuals, herein identified by internal reference numbers C004, C005,

C008 (0.89 to 1.06 fold). The values shown are reciprocal values according to the formula described herein (see below).

EXAMPLE I:

(i) Brain tissue dissection from patients with Alzheimer's disease:

Brain tissues from Alzheimer's disease patients and age-matched control subjects were collected within 6 hours post-mortem and immediately frozen on dry ice. Sample sections from each tissue were fixed in paraformaldehyde for histopathological confirmation of the diagnosis. Brain areas for differential expression analysis were identified (see Figure 1) and stored at -80 °C until RNA extractions were performed.

(ii) Isolation of total mRNA:

Total RNA was extracted from post-mortem brain tissue by using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The accurate RNA concentration and the RNA quality were determined with the DNA LabChip system using the Agilent 2100 Bioanalyzer (Agilent Technologies). For additional quality testing of the prepared RNA, i.e. exclusion of partial degradation and testing for DNA contamination, specifically designed intronic GAPDH oligonucleotides and genomic DNA as reference control were used to generate a melting curve with the LightCycler technology as described in the manufacturer's protocol (Roche).

(iii) cDNA synthesis and identification of differentially expressed genes by suppressive subtractive hybridization:

This technique compares two populations of mRNA and provides clones of genes that are expressed in one population but not in the other. The applied technique was described in detail by Diatchenko et al. (*Proc.*

Natl. Acad. Sci. USA 1996, 93: 6025-30). In the present invention, mRNA populations from post-mortem brain tissues from Alzheimer's disease patients were compared. Specifically, mRNA of the frontal cortex was subtracted from mRNA of the inferior temporal cortex. The necessary reagents were taken from the PCR-Select cDNA subtraction kit (Clontech), and all steps were performed as described in the manufacturer's protocol. Specifically, 2µg mRNA each were used for first-strand and second-strand cDNA synthesis. After RsaI-digestion and adaptor ligation hybridization of tester and driver was performed for 8 hours (first hybridization) and 15 hours (second hybridization) at 68 °C. Two PCR steps were performed to amplify differentially expressed genes (first PCR: 27 cycles of 94 °C and 30 sec, 66 °C and 30 sec, and 72 °C and 1.5 min; nested PCR: 12 cycles of 94 °C and 30 sec, 66 °C and 30 sec, and 72 °C and 1.5 min) using adaptor specific primers (included in the subtraction kit) and 50x Advantage Polymerase Mix (Clontech). Efficiencies of RsaI-digestions, adaptor ligations and subtractive hybridizations were checked as recommended in the kit. Subtracted cDNAs were inserted into the pCR vector and transformed into *E.coli* INVαF' cells (Invitrogen).

To isolate individual cDNAs of the subtracted library, single bacterial transformants were incubated in 100 µl LB (with 50 µg/ml ampicillin) at 37 °C for at least 4 hours. Inserts were PCR amplified (95 °C and 30 sec, 68 °C and 3 min for 30 cycles) in a volume of 20 µl containing 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl₂, 50 mM KCl, 200 µM dNTP, 0.5 µM adaptor specific primers (included in the subtraction kit), 1.5 Units Taq polymerase (Pharmacia Biotech), and 1µl of bacterial culture.

1.5 µl of a mixture containing 3 µl PCR amplified inserts and 2 µl 0.3 N NaOH/15 % Ficoll were spotted onto a positively charged nylon membrane (Roche). In this way, hundreds of spots were arrayed on duplicate filters for subsequent hybridization analysis. The differential screening step consisted of hybridizations of the subtracted library with itself to minimize background (Wang and Brown, *Proc. Natl. Acad. Sci. USA* 1991, 88: 11505-9). The probes were generated from the nested

PCR product of the subtraction following the instructions of the Clontech subtraction kit. Labeling with digoxigenin was performed with the DIG DNA Labeling Kit (Roche). Hybridizations were carried out overnight in DIG Easy HYB (Roche) at 43 °C. The filters were washed twice in 2 x SSC / 0.5 % SDS at 68 °C for 15 min and twice in 0.1 x SSC / 0.5 % SDS at 68 °C for 15 min, and subjected to detection using anti-DIG-AP conjugates and CDP-Star as chemiluminescent substrate according to the instructions of the DIG DNA Detection Kit (Roche). Blots were exposed to Kodak Biomax MR chemiluminescent film at room temperature for several minutes. The nucleotide sequences of clones of interest were obtained using methods well known to those skilled in the art. For nucleotide sequence analyses and homology searches, computer algorithms of the University of Wisconsin Genetics Computer Group (GCG) in conjunction with publicly available nucleotide and peptide sequence information (Genbank and EMBL databases) were employed. The results of one such subtractive hybridization experiment for the SCN2A gene are shown in Figure 2.

(iv) Confirmation of differential expression by quantitative RT-PCR:

Positive corroboration of differential expression of the gene coding for SCN2A was performed using the LightCycler technology (Roche). This technique features rapid thermal cycling for the polymerase chain reaction as well as real-time measurement of fluorescent signals during amplification and therefore allows for highly accurate quantification of RT-PCR products by using a kinetic, rather than an endpoint readout. The ratios of SCN2A cDNA from the temporal cortex and frontal cortex, and from the hippocampus and frontal cortex, respectively, were determined (relative quantification).

First, a standard curve was generated to determine the efficiency of the PCR with specific primers for the gene coding for SCN2A:

5'- TGCAGCAAACAAGGAAGAGCT -3' and

5'-CGGGCTTTTCATCATTGAGTG 3'.

PCR amplification (95 °C and 1 sec, 56 °C and 5 sec, and 72 °C and 5 sec) was performed in a volume of 20 µl containing Lightcycler-FastStart DNA Master SYBR Green I mix (contains FastStart Taq DNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, SYBR Green I dye, and 1 mM MgCl₂, Roche), additional 3 mM MgCl₂, 0,5 µM primers, and 2 µl of a cDNA dilution series (final concentration of 40, 20, 10, 5, 1 and 0.5 ng human total brain cDNA, Clontech). Melting curve analysis revealed a single peak at approximately 78.7°C with no visible primer dimers. Quality and size of the PCR product were determined with the DNA LabChip system (Agilent 2100 Bioanalyzer, Agilent Technologies). A single peak at the expected size of 74 bp for the SCN2A gene was observed in the electropherogram of the sample.

In an analogous manner, the PCR protocol was applied to determine the PCR efficiency of a set of reference genes which were selected as a reference standard for quantification. In the present invention, the mean value of five such reference genes was determined: (1) cyclophilin B, using the specific primers 5'-ACTGAAGCACTACGGGCCTG-3' and 5'-AGCCGTTGGTGTCTTTGCC-3' except for MgCl₂ (an additional 1 mM was added instead of 3 mM). Melting curve analysis revealed a single peak at approximately 87 °C with no visible primer dimers. Agarose gel analysis of the PCR product showed one single band of the expected size (62 bp). (2) Ribosomal protein S9 (RPS9), using the specific primers 5'-GGTCAAATTTACCCTGGCCA-3' and 5'-TCTCATCAAGCGTCAGCAGTTC-3' (exception: additional 1 mM MgCl₂ was added instead of 3 mM). Melting curve analysis revealed a single peak at approximately 85°C with no visible primer dimers. Agarose gel analysis of the PCR product showed one single band with the expected size (62 bp). (3) beta-actin, using the specific primers 5'-TGGAACGGTGAAGGTGACA-3' and 5'-GGCAAGGGACTTCCTGTAA-3'. Melting curve analysis revealed a single peak at approximately 87°C with no visible primer dimers. Agarose gel analysis of the PCR product showed one single band with the expected size (142 bp). (4) GAPDH, using the specific primers 5'-

CGTCATGGGTGTGAACCATG-3' and 5'-GCTAAGCAGTTGGTGGTGCAG-3'. Melting curve analysis revealed a single peak at approximately 83°C with no visible primer dimers. Agarose gel analysis of the PCR product showed one single band with the expected size (81 bp). (5) Transferrin receptor TRR, using the specific primers 5'-GTCGCTGGTCAGTTCGTGATT-3' and 5'-AGCAGTTGGCTGTTGTACCTCTC-3'. Melting curve analysis revealed a single peak at approximately 83°C with no visible primer dimers. Agarose gel analysis of the PCR product showed one single band with the expected size (80 bp).

For calculation of the values, first the logarithm of the cDNA concentration was plotted against the threshold cycle number C_t for the gene coding for SCN2A and the five reference standard genes. The slopes and the intercepts of the standard curves (i.e. linear regressions) were calculated for all genes. In a second step, cDNA from temporal cortex and frontal cortex, and from hippocampus and frontal cortex, respectively, were analyzed in parallel and normalized to cyclophilin B. The C_t values were measured and converted to ng total brain cDNA using the corresponding standard curves:

$$10^{((C_t \text{ value} - \text{intercept}) / \text{slope})} \quad [\text{ng total brain cDNA}]$$

The values for temporal and frontal cortex SCN2A cDNAs, and the values for hippocampus and frontal cortex SCN2A cDNAs, respectively, were normalized to cyclophilin B, and the ratios were calculated according to formulas:

$$\text{Ratio} = \frac{\text{SCN2A temporal [ng]} / \text{cyclophilin B temporal [ng]}}{\text{SCN2A frontal [ng]} / \text{cyclophilin B frontal [ng]}}$$

$$\text{Ratio} = \frac{\text{SCN2A hippocampus [ng]} / \text{cyclophilin B hippocampus [ng]}}{\text{SCN2A frontal [ng]} / \text{cyclophilin B frontal [ng]}}$$

In a third step, the set of reference standard genes was analyzed in parallel to determine the mean average value of the temporal to frontal ratios, and of the hippocampal to frontal ratios, respectively, of expression levels of the reference standard genes for each individual brain sample. As cyclophilin B was analyzed in step 2 and step 3, and the ratio from one gene to another gene remained constant in different runs, it was possible to normalize the values for SCN2A to the mean average value of the set of reference standard genes instead of normalizing to one single gene alone. The calculation was performed by dividing the respective ratio shown above by the deviation of cyclophilin B from the mean value of all housekeeping genes.

The results of such quantitative RT-PCR analysis for the SCN2A gene are shown in Figures 3 and 4.

(v) Immunohistochemistry:

For immunofluorescence staining of the voltage-gated sodium channel SCN2A in human brain, frozen sections were prepared from post-mortem pre-central gyrus of a donor person (Cryostat Leica CM3050S) and fixed in acetone for 10 min. After washing in PBS, the sections were pre-incubated with blocking buffer (10% normal goat serum, 0.2% Triton X-100 in PBS) for 30min, and then incubated with anti-SCN2A mouse monoclonal antibodies (1:40 diluted in blocking buffer, Upstate, Waltham) overnight at 4°C. After rinsing three times in 0.1% Triton X-100/PBS, the sections were incubated with FITC-conjugated goat anti-mouse IgG (1:150 diluted in 1% BSA/PBS) for 2 hours at room

temperature, and then again washed in PBS. Staining of the nuclei was performed by incubation of the sections with 5 μ M DAPI in PBS for 3min (blue signal). In order to block the autofluorescence of lipofuscin in human brain, the sections were treated with 1% Sudan Black B in 70% ethanol for 2-10 min at room temperature, sequentially dipped in 70% ethanol, distilled water and PBS. The sections were coverslipped by 'Vectrashield mounting medium' (Vector Laboratories, Burlingame, CA) and observed under an inverted microscope (IX81, Olympus Optical). The digital images were captured with the appropriate software (AnalySiS, Olympus Optical).